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Although bone matrix derivatives have been shown to induce new bone formation, little is known about the mechanism of matrix-induced osteogenesis. We produced antisera and monoclonal antibodies reactive with bovine bone morphogenetic protein (bBMP) and applied them to tissue sections alone and in combination with histochemical and autoradiographic methods. In this way we studied the in vivo fate of immunologically defined components of the osteoinducer and analyzed their tissue distribution relative to proliferative and enzymatic responses associated with new bone formation. Two monoclonal antibodies, reactive with bBMP in tissue sections, revealed diffusion of the relevant determinant from implanted matrix particles and its incorporation by responding cells. A method for simultaneously demonstrating immunoreactive bBMP, DNA replication and alkaline phosphate activity in the responding cells was shown to result in effective differential labeling of these features in mildly fixed tissue sections. Application of this method with monoclonal antibodies specific for induction-associated determinants and with modifications to permit ultrastructural analyses may provide important information relevant to the mechanism of matrix-induced bone formation.

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Final Report
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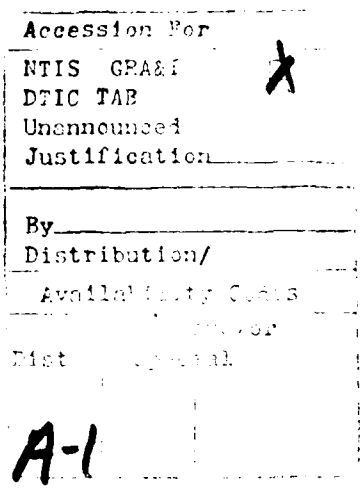
Paul R. Burnett and James W. Mizgala



Pathology Branch

U.S. Army Institute of Dental Research

Washington, DC 20307-5300

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ABSTRACT

Although bone matrix derivatives have been shown to induce new bone formation, little is known about the mechanism of matrix-induced osteogenesis. We produced antisera and monoclonal antibodies reactive with bovine bone morphogenetic protein (bBMP) and applied them to tissue sections alone and in combination with histochemical and autoradiographic methods. In this way we studied the in vivo fate of immunologically defined components of the osteoinducer and analyzed their tissue distribution relative to proliferative and enzymatic responses associated with new bone formation. Two monoclonal antibodies, reactive with bBMP in tissue sections, revealed diffusion of the relevant determinant from implanted matrix particles and its incorporation by responding cells. A method for simultaneously demonstrating immunoreactive bBMP, DNA replication, and alkaline phosphate activity in the responding cells was shown to result in effective differential labeling of these features in mildly fixed tissue sections. Application of this method with monoclonal antibodies specific for induction-associated determinants and with modifications to permit ultrastructural analyses may provide important information relevant to the mechanism of matrix-induced bone formation.
KEY WORDS: Matrix-induced osteogenesis, Bone morphogenetic protein, Monoclonal antibodies, Immunohistochemistry, Autoradiography, Alkaline phosphatase, Simultaneous localization.

INTRODUCTION

There is ample evidence that extracellular matrix glycoproteins regulate the differentiation and function of bone forming cells (1-10). They reproducibly initiate a cascade of cellular and molecular events leading to the formation of mature cartilage and/or bone (1,2,7). When implanted extraskeletally in allogeneic or xenogeneic recipients, these events include the augmentation of DNA replication and increased alkaline phosphatase activity in mesenchymal cells responding to the osteoinductive signal (10,11,12). These activities appear to be dependent upon the presence of both a collagenous matrix substratum and a complex mixture of extractable matrix proteins (3,4,5,10). Such materials are currently being employed as models to study cellular developmental processes in general and osteogenesis in particular and as experimental therapeutic agents for the treatment of certain metabolic diseases and traumatic injuries to bone. However, the nature of the molecular interactions between these complex materials and responding osteocompetent mesenchymal cells remains poorly understood.

In an attempt to elucidate these interactions by an immunohistochemical approach, we have produced a number of different monoclonal antibodies to a partially purified osteoinductive matrix preparation. We then developed a method for applying these antibodies to tissue sections in conjunction with autoradiographic and enzyme histochemical procedures to simultaneously

analyze the association of proliferative and elevated alkaline phosphatase responses with specific immunoreactive determinants of the complex morphogen.

MATERIALS AND METHODS

Implants

Partially purified bovine Bone Morphogenetic Protein (bBMP) was obtained from the UCLA Bone Research Laboratory. When subjected to polyacrylamide gel electrophoresis under denaturing conditions, this material showed several distinct bands ranging from 15-75 kilodaltons. Doses ranging from 5 to 10 mg were measured into gelatin capsules which in turn were heat sealed in nylon mesh chambers to minimize dispersion of the material in vivo and simplify retrieval of representative tissue specimens. Control implants lacking bBMP were packaged in the same manner, and all implants were subjected to ethylene oxide sterilization.

Tissue Specimens

To minimize the inductive effects of endogenous bone matrix proteins and simplify subsequent tissue processing procedures, this study was limited to analyses of extraskeletal implants placed in the pectoral muscle mass of Sprague-Dawley rats. Animals were treated in compliance with the Animal Welfare Act and principles stated in the "Guide for the Care and Use of Laboratory Animals," in NIH publication 85-23.

Sixteen hours prior to CO_2 asphyxiation, implant-bearing rats received an intraperitoneal injection of 1 μCi (specific activity 20 Ci/mmol) per gram body weight of [methyl-^{3}H] thymidine (New England Nuclear, Boston, MA). Postmortem tissue specimens were retrieved, washed and fixed for 2 hours in cold, neutralbuffered

2% formaldehyde, and dehydrated in a graded ethanol series in preparation for embedment in paraffin. The temperature of the molten paraffin was not allowed to exceed 56°C, and total infiltration and embedment time did not exceed 15 minutes.

Alternatively, selected specimens were quenched in liquid nitrogen-cooled 2-methyl butane and dehydrated overnight in a vacuum tissue drier (Edwards High Vacuum Inc., Grand Island, NY) at -40°C, prior to embedment in paraffin. This technique has been reported to yield sections with good perservation of architectural detail and immunoreactivity of formaldeyde-labile determinants (13,14). All blocks and serial 5 μm sections were stored in a -20° freezer to minimize depression of alkaline phosphatase activity.

Immunohistochemistry

Primary antibodies. Polyclonal anti-bBMP sera were obtained by exsanguination of Balb/cbyJ mice which had been immunized with bBMP (0.5 mg/ml) in Freund's complete adjuvant emulsion. Sera found to be reactive with bBMP by enzyme-linked immunosorbent assay (ELISA) yielded optimal results when applied to the mildly fixed tissue sections at dilutions ranging from 1:50 to 1:400. Monoclonal antibodies specific for determinants of bBMP were prepared by fusing spleen cells from the bBMP-seropositive mice with non-secreting P3-X63-Ag8.653 mouse myeloma cells (15). Conventional hybridoma methodology based on the techniques originally described by Kohler and Milstein (16) was employed.

The monoclonal antibodies, derived from clones of a single fused cell and determined to be reactive with bBMP by ELISA, were incubated with tissue sections at dilutions of the hybridoma supernatants ranging from 1:10 to 1:80.

Immunoperoxidase labeling. Sections previously incubated with bMBP-specific antibodies or control sera were labeled for brightfield microscopy by the avidin-biotin-peroxidase complex (ABC) method (17) using kits including rat-adsorbed biotinylated anti-mouse IgG serum (Vector Laboratories, Burlingame, CA) as the secondary antibody and 3,3' diaminobenzidine (Sigma, St. Louis, MO) as the chromogen. Mayer's hematoxylin was employed as a counterstain.

Alkaline phosphate histochemistry

The Burstone method (18) at pH 8.6 was used to demonstrate alkaline phosphatase activity in tissue sections. Commercially acquired reagents (Sigma) included napthol AS-MX phosphate acid as substrate, N.N. dimethyl-formamide, and fast red violet LB salt as chromogen. Specificity controls comprised parallel sections which had been subjected to 100°C or reacted in the absence of substrate or at acid pH.

Autoradiography

Tissue sections to be analyzed for [^{3}H] thymidine incorporation were processed for light-microscopic autoradiography by conventional methods (19). Following rehydration they were dipped in NTB-2 nuclear track emulsion (Eastman Kodak, Rochester,

NY) allowed to dry, and exposed in light-proof boxes with desiccant at 4°C for 7 days. At the end of the exposure period the emulsions were processed in Dektol developer (Kodak) for 2 min., treated with Kodak fixer for 6 min., and washed thoroughly prior to counterstaining with Mayers hematoxylin. Controls included sections from both [^{3}H]-free bBMP-positive specimens and [^{3}H]-exposed bBMP-negative specimens.

Multiple labeling procedures

In order to simultaneously demonstrate immunoreactive inducer and biochemical responses, combinations of two or all three of the above described labeling methods and counterstain were performed in different sequences. Stain compatibility was evaluated as a function of the order of their application. Qualitative analyses were made of the relative quantity and location of the individually demonstrated features as a function of the presence or absence of bBMP and the period of implantation. The efficiency of the individual components of multiple labeling procedures was rated by comparison to the corresponding single-labeled sections.

RESULTS

Monoclonal Anitbodies to bBMP. From three successful cell-fusion procedures, we obtained a total of 38 hybrid clones producing antibodies reactive with the bBMP immunogen by ELISA. Of these, only two were found to be reactive with bBMP in mildly fixed tissue sections. On subsequent testing, several of the bBMP-positive monoclonal antibodies found to be non-reactive with fixed tissue sections were also found to be non-reactive with both freeze-dried and conventional frozen sections containing bBMP.

Immunohistochemistry

Labeling of bBMP-containing tissue sections with polyclonal antisera or selected monoclonal antibodies to bBMP revealed a time-dependent decoration of the implanted matrix particles and responding ovoid and spindle-shaped cells. Immunoreactivity in implants retrieved four days postimplantation was limited to the matrix particles per se. Polyclonal anti-bBMP labeling of implants retrieved between 5-8 days postimplantation demonstrated a progressive decoration of the cytoplasm of selected responding cells, in addition to the labeling of the matrix particles. However, labeling of the same specimens with a monoclonal antibody to bBMP revealed, in addition to the progressive labeling of the responding cells, a simultaneous loss of matrix reactivity, such that by day 8 all immunoreactivity resided in the cytoplasm of the responding cells (Fig. 1). None of the negative control

sections showed any bBMP-positive labeling.

Alkaline Phosphatase Histochemistry

Specimens retrieved 4-12 days postimplantation demonstrated time-dependent, temperature-sensitive labeling of spindle and ovoid cells adjacent to the implanted matrix particles (Figs. 2 and 3) and rimming newly formed chondroid and osteoid matrix. Little or no AP activity was detected in specimens lacking bBMP, and none of the negative control sections demonstrated significant AP activity. No AP activity was found in any sections exposed to aqueous solutions or room temperature for more than a few hours prior to labeling.

[^{3}H] thymidine Autoradiography

Specimens retrieved 3-12 days postimplantation demonstrated time-dependent [^{3}H] thymidine-labeling of cells adjacent to implanted matrix particles (Fig. 3) nearby vascular spaces, and new matrix. The number of labeled nuclei was greatest at 3-6 days postimplantation. Although labeling was noted on the periphery of newly formed cartilage, no labeling of differentiated chondrocytes within the newly formed matrix was observed. Exposure times of about 7 days appeared to be optimal for revealing specifically labeled nuclei while holding background fog at an acceptable level.

Multiple-Labeling Procedures

Burstone's method for alkaline phosphatase and the dipping emulsion method for demonstrating [^{3}H] thymidine were individual-

ly and jointly found to be compatible with the ABC immunoperoxidase method for labeling antigenic determinants in mildly fixed tissue sections. The satisfactory demonstration of each of the individual components, however, was dependent upon application of the the various procedures in the proper sequence: alkaline phosphatase method followed by immunoperoxidase labeling, emulsion coating, autoradiographic exposure, and counterstaining, in that order. Dual-labeling for immunoreactive bBMP and alkaline phosphatase resulted in easily distinguishable populations of single-labeled cells (Fig. 2); however, there were no unequivocally dual-labeled cells in the sections examined. The triple labeling method revealed populations of single-labeled AP-positive or [^{3}H] thymidine-positive cells as well dual AP and [^{3}H] thymidine-labeled cells and demonstrated their spatial association with immunoreactive bBMP (Fig. 3). Application of this method to specimens retrieved at different intervals postimplantation revealed an initial [^{3}H] thymidine-labeling of some of the responding cells by day 3, followed within 24-48 hours by the appearance of a greater number of AP-positive cells which subsequently were intimately associated with foci of new cartilage and bone formation.

DISCUSSION

Immunohistochemistry

We have shown that monoclonal antibodies reactive with bone matrix macromolecules can be produced and applied to mildly fixed tissue sections to study the postimplantation fate of these natural osteoinducers. The failure of most of the bBMP reactive monoclonals to react with the immunogen in aldehyde-fixed 3-12 day implant specimens may be primarily a reflection of the degradation of native determinants in vivo. A similar failure of several of these antibodies to label the immunogen in freeze-dried and conventional frozen sections suggests that this nonreactivity was not solely the result of the fixation process. Undoubtly, however, with the production and testing of more monoclonal antibodies to bBMP, some will be found to be reactive only with unfixed sections. Therefore the freeze drying method of tissue preparation (13,14), followed by nondestructive embedding in a plastic resin (20) to permit both light microscopic and ultrastructural analyses, may prove to be a more generally applicable method.

Application of the two bBMP-specific monoclonals, which were found to react with fixed tissue sections, demonstrated the diffusion of a bBMP determinant from the implanted matrix particles, and its incorporation by responding cells. This observation is consistent with, but not conclusive evidence of, the transfer of an inductive signal from the matrix preparation to

responding osteocompetent cells. The precise specificity of the antibodies and the identity of the labeled cells remain to be determined. An attempted investigation of the possible phagocytic nature of these cells by immunohistochemical labeling with primary antibodies specific for enzyme and surface membrane markers (21,22) has yielded equivocal results due to the ambiguous staining of control sections. Ultrastructural analysis should prove more helpful in establishing their identity.

Autoradiography

Our results confirm previously reported findings (11,12) of a proliferative response to extraskeletally-implanted bone matrix, with labeled nuclei localized to mesenchymal cells adjacent to the matrix particles and foci of newly formed cartilage or bone. Furthermore, we have confirmed the reported compatibility of the dipping emulsion method for detecting [^{3}H] thymidine incorporation with alkaline phosphatase labeling histochemistry (23). This method was used to demonstrate a proliferative response preceding amplification of alkaline phosphatase activity and the subsequent appearance of chondroid and osteoid matrix.

Multiple Labeling Procedures

We have shown that by the combined application of immunohistochemical, enzyme histochemical and autoradioghic procedures, one can simultaneously demonstrate proliferative and enzymatic responses to multideterminant osteoinducers and relate them to the tissue distribution of a single immunoreactive determinant.

Our analysis of these prepartions is presently complicated by our uncertainty as to the relative sensitivities of the various labels. For example, the detection of [^{3}H] thymidine labeling prior to the demonstrated incorporation of immunreactive bBMP by responding cells could be a reflection of our inability to detect the initial uptake by these cells of minute quantities of the inducer, and not a true indicator of the order of these events. Nonetheless, we believe that when employed with a monoclonal antibody of proven specificity for an induction-associated determinant and modified to incorporate increasingly sensitive immunoperoxidase-labeling techniques, the method described here will yield important information relevant to the mechanism of matrix-induced bone formation.

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Legends

Figure 1. Eight day bBMP implant labeled with monoclonal antibody to bBMP. Section shows cytoplasmic decoration (golden-brown chromogen) of selected ovoid and spindle-shaped cells adjacent to unlabeled particles of implanted matrix. Bar = 25 um.

Figure 2. Eight day bBMP implant labeled initially for alkaline phosphatase (AP) activity (red chromogen) and then for reactivity with a monoclonal antibody to bBMP (golden-brown chromogen). Section shows unequivocal and easily distinguishable labeling of AP-positive and bBMP-positive populations of cells adjacent to unlableled matrix particle. Bar = 25 um.

Figure 3. Eight day bBMP implant labeled sequentially for alkaline phosphatase (AP) activity (red chromogen), immunoreactivity with polyclonal anti-bBMP (golden-brown chromogen) and [^{3}H] thymidine incorporation (black grains). Section shows bBMP-positive decoration of implanted matrix particles and adjacent ovoid-shaped cells, as well as associated populations of AP-positive cells, [^{3}H] thymidine-labeled cells and dual AP-positive, [^{3}H] thymidine-labeled cells. Bar = 25 um.

Fig. 1

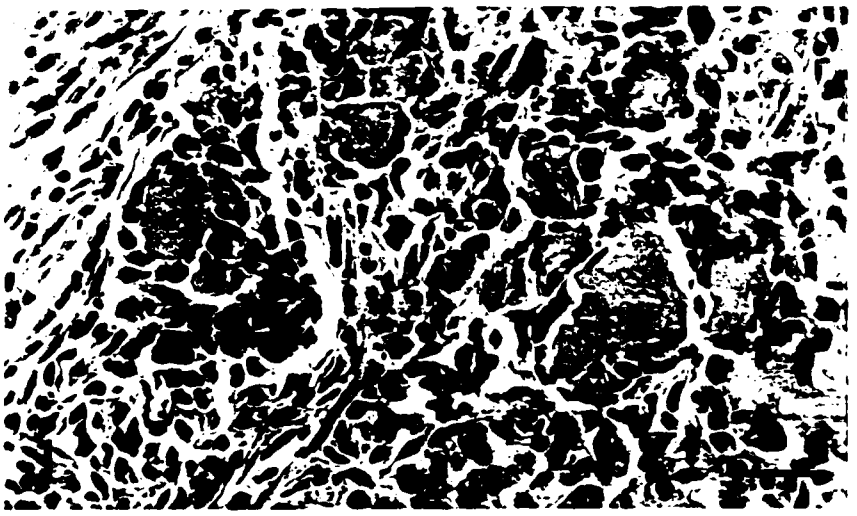

Fig. 2

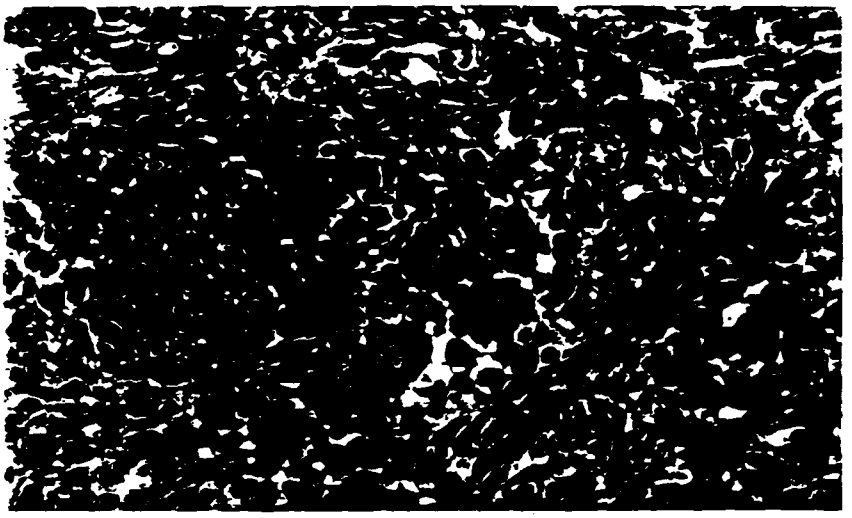

Fig. 3